Brief Report

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Docosahexaenoic acid increases tyrosine hydroxylase phosphorylation at Ser40 without increasing tyrosine hydroxylase protein expression in differentiated NG108-15 cells

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SUMMARY: Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine biosynthesis, and its activity is regulated by its phosphorylation at specific serine (Ser) residues. In the present study, we investigated the effects of docosahexaenoic acid (DHA) supplementation on TH protein expression and phosphorylation at Ser31 and Ser40 during differentiation in the neuroblastoma–glioma hybrid cell line NG108-15. TH protein expression and phosphorylation levels were analyzed on day 0 (undifferentiated) and on days 5 and 6 (differentiated). Differentiation increased TH protein expression on days 5 and 6, and DHA supplementation did not affect these increases. Phosphorylation at Ser31 showed no significant change with differentiation. By contrast, phosphorylation at Ser40 exhibited a significant increase with increasing days of differentiation, and this was further augmented by DHA treatment. Collectively, these findings suggest that DHA enhances TH phosphorylation, particularly at Ser40, without affecting TH protein expression. DHA may therefore influence brain function not through changes in TH expression levels, but rather through the phosphorylation of TH at Ser40.

Keywords: DHA, n-3 polyunsaturated fatty acids, neuronal cell, tyrosine hydroxylase, tyrosine hydroxylase phosphorylation at Ser 40

1. Introduction

Tyrosine hydroxylase (TH) catalyzes the hydroxylation of tyrosine to L-3,4 dihydroxyphenylalanine and is the rate-limiting enzyme in catecholamine synthesis (*1,2*). The catecholamines dopamine, noradrenaline, and adrenaline play roles in many brain functions as well as in neuronal diseases and disorders (*3-5*).

TH contains serine (Ser) residues at Ser8, Ser19, Ser31, and Ser40 that can be phosphorylated by a variety of protein kinases. It is thought that the phosphorylation of Ser8 and Ser19 has no direct effect on TH enzymatic activity (*6,7*), whereas the phosphorylation of Ser31 and Ser40 directly regulates TH activity (*8*).

Polyunsaturated fatty acids are important constituents of mammalian phospholipids. Docosahexaenoic acid (DHA) is abundant in the central nervous system as a component of phospholipids and is reportedly involved in various brain functions including neuronal outgrowth, synaptic plasticity, mood regulation, learning, and memory (*9-14*). In the striatum of rats treated with 6-hydroxydopamine (6-OHDA), Ser40 phosphorylation and TH expression levels decrease; however, DHA treatment suppresses this decrease (*15*). By contrast, DHA does not alter Ser40 phosphorylation or TH expression in rats not treated with 6-OHDA (*15*).

NG108-15 cells are a neuroblastoma–glioma hybrid cell line that exhibit neuronal-like morphology and properties when differentiated (*16,17*). In the present study, we used NG108-15 cells to examine the effects of adding DHA to differentiation-inducing medium on TH protein expression and TH phosphorylation at Ser31 and Ser40.

2. Materials and Methods

2.1. Materials

NG108-15 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture medium and dexamethasone were purchased from Fujifilm Wako (Osaka, Japan). Dibutyryl cyclic adenosine monophosphate was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Nacalai

Tesque (Kyoto, Japan). Hypoxanthine, aminopterin, and thymidine (HAT) supplement (50×) was purchased from MP Biomedicals (Santa Ana, CA, USA). DHA was purchased from Cayman (Ann Arbor, MI, USA).

2.2. Cell culture

NG108-15 cells were grown and maintained in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2. Figure 1 shows the study design. Cells were seeded in 12-well plates at 5000 cells/cm^2. After 24 hours, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, HAT, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μM α-tocopherol, 0.2 mM dibutyryl cyclic adenosine monophosphate, and 100 nM dexamethasone, which was added to induce differentiation (*16,17*). Additionally, DHA (2 μM) bound to 0.05% fatty acid–free bovine serum albumin (BSA) was added to medium in the DHA(+) group. The medium for the DHA(−) group contained 0.05% fatty acid–free BSA without DHA. The cells were then cultured for 5 or 6 days.

2.3. Preparation of samples for western blot analysis

Cells were harvested in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2 ethylenediaminetetraacetic acid, 1 mM egtazic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and the samples were sonicated. The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) using BSA as the standard (*18*).

Aliquots were then mixed with concentrated sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis sample buffer (final concentrations: 62.5 mM Tris-HCl, pH 6.8, 2% 2-mercaptoethanol, 10% glycerol, 2% SDS, and 0.01% bromophenol blue)).

2.4. Western blot analysis

For SDS polyacrylamide gel electrophoresis, samples containing equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels and subsequently transferred to polyvinylidene fluoride membranes (*19,20*). The membranes were blocked with polyvinylidene fluoride blocking reagent (Toyobo, Tokyo, Japan) before being incubated overnight at 4°C with the following primary antibodies: TH (#2792, Cell Signaling Technology, Danvers, MA, USA), phosphorylated-TH (Ser31) (#13041, Cell Signaling Technology), phosphorylated-TH (Ser40) (#2791, Cell Signaling Technology), and β-actin (A5441, Sigma). The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies (Dako, Glostrup, Denmark) and developed using SuperSignal West Pico (Thermo Fisher Scientific, Waltham, MA, USA) or ImmunoStar LD reagents (Fujifilm Wako). Signal detection and band intensity quantification were performed using an Amersham Imager 680 (Cytiva, Tokyo, Japan).

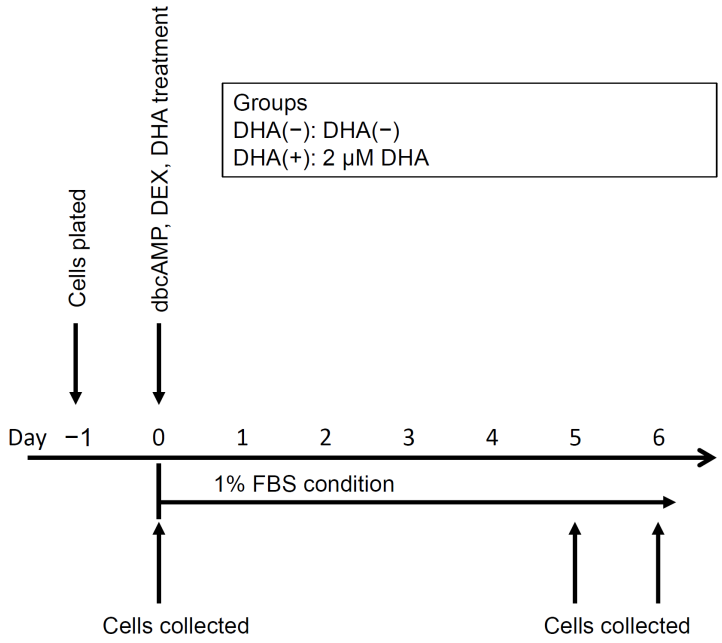

Figure 1. Cell culture and treatment. The day after seeding cells (day 0), differentiation medium (including dbcAMP and DEX) and/or DHA were added. On days 0, 5, and 6, cells were collected. dbcAMP, dibutyryl cyclic AMP; DEX, dexamethasone; DHA, docosahexaenoic acid.

2.5. Statistical analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$. Excel-Toukei software (2012, Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for the statistical analysis.

3. Results and Discussion

We investigated TH protein expression and phosphorylation on day 0 (undifferentiated) and days 5 and 6 (differentiated) to determine the effects of DHA on NG108-15 cells.

Figure 2 shows TH protein expression in NG108-15 cells after incubation for 5 or 6 days in differentiation-inducing medium. In the DHA(−) and DHA(+) groups, TH protein expression was increased with increasing days of differentiation. There was no significant difference between the groups with and without DHA addition.

Figure 3 shows TH phosphorylation at Ser31 in NG108-15 cells after incubation for 5 or 6 days in differentiation-inducing medium. There were no significant effects of days of differentiation, DHA treatment, or their interaction. However, in the DHA(+) group, a trend toward an increase was observed compared with the DHA(−) group.

Figure 4 shows TH phosphorylation at Ser40 in NG108-15 cells after incubation for 5 or 6 days in differentiation-inducing medium. TH phosphorylation at Ser40 was increased with increasing days of differentiation ($P < 0.005$), and the level was greater in the DHA(+) group than in the DHA(−) group. The level of TH phosphorylation at Ser40 in the DHA(+) group was increased by approximately 1.4-fold on day 5 and 2.2-fold on day 6 compared with the DHA(−) group.

It has been reported that TH is phosphorylated at Ser31 by both extracellular signal-regulated kinase1/2 and cyclin-dependent kinase 5 *in vitro* and *in vivo* (*21,22*). By contrast, TH is reportedly phosphorylated at Ser40 by cyclic AMP–dependent protein kinase (protein kinase A; PKA), protein kinase C, or protein kinase G *in vitro* and *in situ*, or by PKA *in vivo* (*21*). However, TH is reportedly dephosphorylated by protein phosphatase PP2A *in vitro* and *in vivo* (*23*). Additionally, *N*-docosahexaenoylethanolamine, a metabolite of DHA, is reported to be synthesized in the brain and activates PKA *via* GPR110 (*24*). In the present study, it is therefore possible that PKA was activated *via* this pathway, resulting in the increased phosphorylation of TH at Ser40.

Differentiation led to increased TH protein expression on days 5 and 6 compared with day 0. These increases were not affected by the addition of DHA (Figure

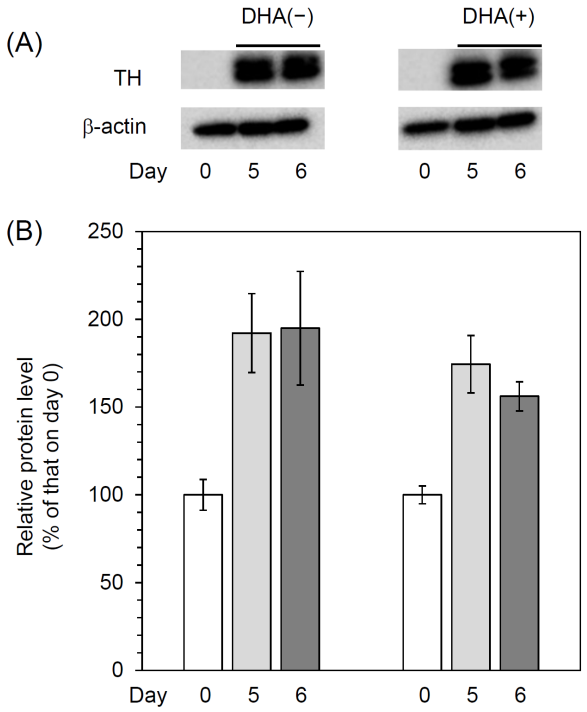

Figure 2. Western blot analysis of TH protein levels in NG108-15 cells on days 0 (undifferentiated), 5, and 6 of differentiation. (**A**) Representative western blots of TH and β-actin. (**B**) Semiquantitative analysis of TH/β-actin. Each column and bar represent the mean and standard error of the mean of four individual experiments. Using two-way ANOVA, there was an effect with days of differentiation ($P < 0.0005$). ANOVA, analysis of variance; DHA, docosahexaenoic acid; TH, tyrosine hydroxylase.

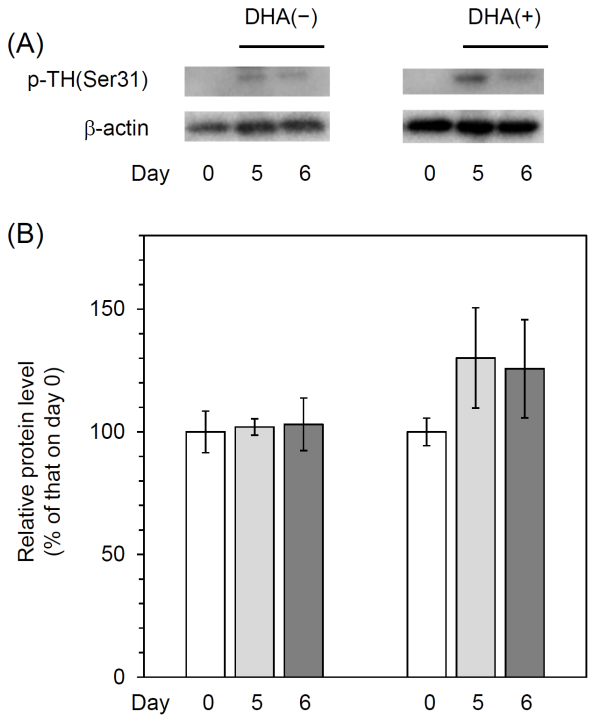

Figure 3. Western blot analysis of TH with Ser31 phosphorylation in NG108-15 cells on days 0 (undifferentiated), 5, and 6 of differentiation. (**A**) Representative western blots of TH with Ser31 phosphorylation and β-actin. (**B**) Semiquantitative analysis of TH with Ser31 phosphorylation/β-actin. Each column and bar represent the mean and standard error of the mean of four individual experiments. DHA, docosahexaenoic acid; p, phosphorylated; TH, tyrosine hydroxylase.

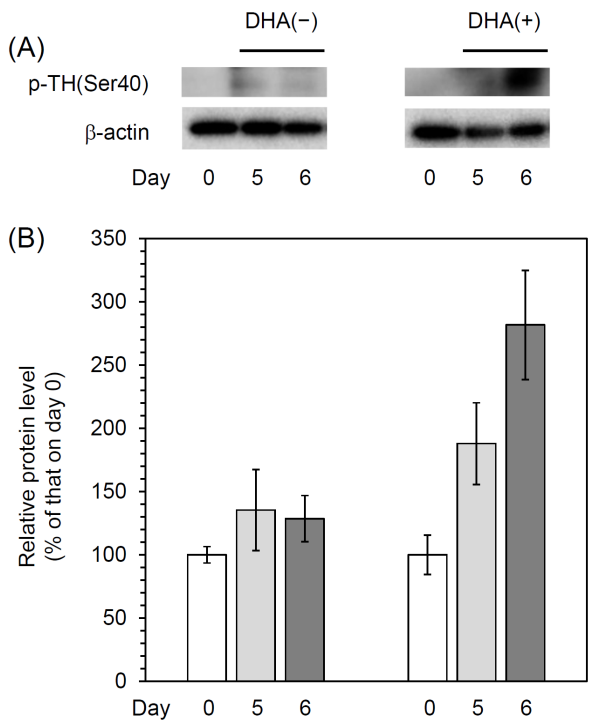

Figure 4. Western blot analysis of TH with Ser40 phosphorylation in NG108-15 cells on days 0 (undifferentiated), 5, and 6 of differentiation. (**A**) Representative western blots of TH with Ser40 phosphorylation and β-actin. (**B**) Semiquantitative analysis of TH with Ser40 phosphorylation/β-actin. Each column and bar represent the mean and standard error of the mean of four individual experiments. Using two-way ANOVA, there were effects with days of differentiation ($P < 0.005$) and DHA treatment ($P < 0.01$) and an interaction between days of differentiation and DHA treatment ($P < 0.05$). ANOVA, analysis of variance; DHA, docosahexaenoic acid; p, phosphorylated; TH, tyrosine hydroxylase.

2). Differentiation did not significantly increase TH phosphorylation at Ser31 on days 5 and 6 compared with day 0; however, a trend toward an increase was observed with the addition of DHA (Figure 3). Phosphorylation at Ser40 was increased with differentiation, and this was enhanced by the addition of DHA (Figure 4).

Under the conditions used in the present study, DHA did not affect TH protein levels; however, TH phosphorylation at Ser31 tended to increase, and TH phosphorylation at Ser40 increased significantly. Although our experiments were performed on cells *in vitro*, the addition of DHA into the medium increased the phosphorylation of TH at Ser40 in neuron-like differentiated NG108-15 cells. Previous studies have suggested that phosphorylation at Ser31 modulates phosphorylation at Ser40 (*25*). Moreover, phosphorylation at Ser40 is more directly involved in the activation of TH compared with the other phosphorylation sites (*8*).

In the striatum of Parkinson's disease model rats treated with 6-OHDA, Ser40 phosphorylation and TH expression levels decrease, and DHA treatment suppresses this decrease (*15*). However, this previous study did not examine Ser31 phosphorylation. Furthermore, DHA treatment in animals not treated with 6-OHDA does not increase Ser40 phosphorylation or TH expression levels (*15*). By contrast, our study indicates that Ser40 phosphorylation, which increases during differentiation induction, is further increased by DHA. Similarly, other studies have demonstrated that adding DHA to NG108-15 cells increases choline acetyltransferase expression, activity, and muscarinic receptors, although they did not examine TH protein expression or its phosphorylation (*16,17*).

In the present study, we examined the effects of DHA on TH expression and phosphorylation during the differentiation of NG108-15 cells. DHA did not alter TH protein levels; however, it increased TH phosphorylation, particularly at Ser40, which is a site that is directly linked to TH activation. Phosphorylation at Ser31 also showed an increasing trend. Together, these findings suggest that DHA may enhance TH activity through intracellular signaling pathways, thereby potentially promoting catecholamine synthesis. Decreased catecholamines are associated with various diseases and disorders including depression, attention deficit hyperactivity disorder, and pure autonomic failure (*3-5*). It has been reported that an increase in Ser40 phosphorylation, induced by phosphodiesterase inhibition and guanylate cyclase-C activation, leads to an improvement in motor deficits in a 6-OHDA-induced Parkinson's disease model (*26*). In summary, the present study provides evidence that DHA selectively promotes the Ser40 phosphorylation of TH without altering the total amount of TH protein in differentiated NG108-15 cells, thereby revealing a possible mechanism by which DHA modulates dopaminergic capacity. We believe that further studies may help to elucidate the relationship between DHA and these diseases and ultimately support their prevention or mitigation.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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